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(54) Title: EXTRACELLULAR POLYPEPTIDES OF EPH B RECEPTORS AND EPHRIN B LIGANDS AND THE CORRE-  
SPONDING NUCLEIC ACID MOLECULES

(57) Abstract: The present invention relates to isolated extracellular polypeptides of Eph B receptors and ephrin B ligands and the corresponding isolated nucleic acid molecules comprising the sequences coding for such polypeptides, to recombinant vectors, such as viral vectors, that comprise such nucleic acid molecules, to host cells comprising such recombinant vectors, to pharmaceutical compositions comprising such polypeptides, nucleic acid molecules or recombinant vectors, and to the use of such vectors in the production of pharmaceutical compositions for treating a mammalian disease, including a human disease, especially tumour diseases and ocular neovascularization, by means of gene therapy.

**Extracellular polypeptides of Eph B receptors and ephrin B ligands and the corresponding nucleic acid molecules**

**Field of the Invention:**

The present invention relates in particular to isolated extracellular polypeptides of Eph B receptors and ephrin B ligands and the corresponding isolated nucleic acid molecules comprising the sequences coding for such polypeptides, to recombinant vectors, such as viral vectors, that comprise such nucleic acid molecules, to host cells comprising such recombinant vectors, to pharmaceutical compositions comprising such polypeptides, nucleic acid molecules or recombinant vectors, and to the use of such vectors in the production of pharmaceutical compositions for treating a mammalian disease, including a human disease especially tumour diseases and ocular neovascularization, by means of gene therapy.

**Background of the Invention:**

Eph receptors with 14 different members form the largest sub-group of receptor tyrosine kinases up to now. The extracellular domain consists of a N-terminal globular domain, a cystein-rich domain and two fibronectin type III domains [Himanen *et al.*, Nature 396, 486-491 (1998)].

Owing to the extracellular domain and ligand recognition, the group is sub-divided into two sub-classes: Eph A (A1-A8) and Eph B (B1-B6) [Eph Nomenclature Committee, Cell 90, 403-404 (1997)].

All of the eight ligands known up to now are proteins attached to membrane and are called ephrins. Here also, two sub-classes may be distinguished: ephrin A ligands (A1-A5) are inserted in the membrane by a glycosyl phosphatidyl inositol anchor (GPI), and ephrin B ligands (B1-B3) have a transmembrane domain and a cytoplasmatic protein section consisting of 82-88 amino acids [Eph Nomenclature Committee, Cell 90, 403-404 (1997); Beckmann *et al.*, EMBO J. 13, 3757-3762 (1994)].

Ephrins of the A class bind predominantly to Eph A receptors and ephrins of the B type to Eph B receptors. Within the classes, however, the ligands have overlapping specificities.

Proteins of the Eph/ephrin family play a role in cell and axon orientation. In contrast to chemoattractive substances, Eph/ephrin interactions in the neuronal and epidermal tissues lead to the production of repulsive signals between the cell populations, which express the corresponding receptors and ligands. Using mice whose genes from the Eph/ephrin family have been specifically inactivated, it could be shown that various Eph/ephrins, in particular from the B class, take part in the development of neural crest and in the development of various regions of the brain. Eph/ephrins are thereby made responsible in particular for the complex topography of neuronal systems, which is controlled by direct cell interaction [Brambilla R. and Klein R., Mol. Cell. Biol. 15, 4921-4929 (1995); Henkemeyer *et al.*, Cell 86, 35-46 (1996)].

Evidence of the participation of the Eph B receptors and ephrin B ligands in embryonal vascular development was provided by genetic experiments. Following homozygote destruction of the ephrin B2 locus, a disturbed formation of the arteries and veins of the capillary vascular system was observed [Wang *et al.*, Cell 93, 741-753 (1998)]. In agreement with this, it was shown that ephrin B2 [GeneBank Acc. Nos.: L38847 (murine), U81262 (human); SwissProt Acc. Nos.: P52800 (murine), P52799 (human)] is expressed primarily on arterial endothelial cells, and on the other hand the receptor Eph B4 [GeneBank Acc. Nos.: U06834 (murine), U07695 (human); SwissProt Acc. Nos.: P54761 (murine), P54760 (human)], which binds ephrin B2, is expressed on venous endothelial cells [Wang *et al.*, Cell 93, 741-753 (1998)]. To confirm this, targeted inactivation of the Eph B4 gene also exhibits a lethal phenotype similar to ephrin B2, as well as the above-described defects in embryonal vascular development [Gerety *et al.*, Molecular Cell 4, 403-414 (1999)]. Further experiments on the function of the Eph B receptor class show, moreover, that Eph B receptors and ephrin B ligands also take part in the cell communication between endothelial cells and the surrounding mesenchymal cells. Mice which cannot express Eph B2 and Eph B3 die *in utero* after about 11.5 days. Analysis of the embryos showed that these animals have defects in the reorganisation of the embryonal vascular system, and exhibited disrupted cell contact between the mesenchymal pericytes and the endodermal endothelial cells [Adams *et al.*, Genes and Dev. 13, 295-308 (1999)].

All previous work on the function of Eph B receptors and ephrin B ligands was, however, restricted to the embryonal development of the nervous system and of the embryonal vascular system. Surprisingly, it has now been discovered for the first time that Eph B

receptors and ephrin B ligands in an adult mammal organism participate functionally in angiogenesis. In addition, it can be shown for the first time that Eph B receptors and ephrin B ligands also take part in the pathophysiology of malignant diseases and that extracellular polypeptides of Eph B receptors and ephrin B ligands are suitable for treating tumour diseases. Moreover, it was found that said polypeptides are also suitable for the treatment of ocular neovascularization. It was also surprisingly established that extracellular polypeptides of Eph B receptors and ephrin B ligands possess pharmacokinetic properties of the type which act not only locally but also systemically and have no evident toxic side effects on the organism of mammals.

**Definitions:**

Within the context of this disclosure the following terms have the meanings as defined below:

In the expression "a soluble polypeptide", the term "soluble" indicates that the respective polypeptide is a non-membrane bound polypeptide which is secreted by mammalian cells which express it. If such a "soluble" polypeptide is expressed for example in cultured mammalian cells, said polypeptide can be found in the cell culture supernatant.

"High affinity" in connection with the binding of ephrin B ligands with Eph B receptors is understood to mean a dissociation constant below 500 nM, preferably in the range of 0.05 nM to 100 nM, more preferably 0.1 nM to 20 nM, the typical range of dissociation constants of ephrin B ligands to Eph B receptors.

The term "essentially similar" used in connection with amino acid sequences refers to amino acid sequence identities. The extracellular regions of different Eph B receptors or ephrin B ligands within the same species have amino acid sequence identities of typically 40% and more among one another. On the other hand, the extracellular regions of the same Eph B receptors or ephrin B ligands of different species typically have amino acid sequence identities of 80% and more. Within the context of this application, a sequence must have at least 40%, preferably at least 75%, most preferably at least 90% amino acid sequence identity to the reference sequence, in order to be regarded as "essentially similar".

**Description of the Invention:**

- i) The Invention relates to an isolated soluble polypeptide which comprises an amino acid sequence of an extracellular region of an Eph B receptor or an essentially similar amino acid sequence thereof, said isolated soluble polypeptide being capable of binding with high affinity to a mammalian ephrin B ligand.
- ii) Similarly the invention also relates to an isolated soluble polypeptide which comprises an amino acid sequence of an extracellular region of an ephrin B ligand or an essentially similar amino acid sequence thereof, said isolated soluble polypeptide being capable of binding with high affinity to a mammalian Eph B receptor.
- iii) The invention further relates especially to an isolated soluble polypeptide which comprises an amino acid sequence of an extracellular region of Eph B4 or an essentially similar amino acid sequence thereof, said isolated soluble polypeptide being capable of binding with high affinity to a mammalian ephrin B ligand.
- iv) Similarly, the invention relates especially to an isolated soluble polypeptide which comprises an amino acid sequence of an extracellular region of ephrin B2 or an essentially similar amino acid sequence thereof, said isolated soluble polypeptide being capable of binding with high affinity to a mammalian Eph B receptor.
- v) An especially preferred embodiment of the invention relates to an isolated soluble polypeptide comprising SEQ ID NO:2 [amino acids (aa) 1-539 of SwissProt Database Accession No. P54761] or SEQ ID NO:4 (aa 1-539 of SwissProt Database Accession No. P54760), which stand for the murine and human extracellular region of Eph B4, respectively, or comprising an amino acid sequence which is essentially similar to SEQ ID NO:2 or SEQ ID NO:4, respectively, said isolated soluble polypeptide being capable of binding with high affinity to a mammalian ephrin B ligand.
- vi) Similarly, a further especially preferred embodiment of the invention relates to an isolated soluble polypeptide which comprises SEQ ID NO:6 (aa 1-229 of SwissProt Database Accession No. P52800) or SEQ ID NO:8 (aa 1-229 of SwissProt Database Accession No. P52799), which stand for the murine and human extracellular region of ephrin B2, respectively, or comprising an amino acid sequence which is essentially similar

to SEQ ID NO:6 or SEQ ID NO:8, respectively, said isolated soluble polypeptide being capable of binding with high affinity to a mammalian Eph B receptor.

vii) The invention also relates to a pharmaceutical composition comprising a polypeptide described in the preceding paragraphs i) – vi), especially such a polypeptide mentioned as being preferred, and to the use of such a polypeptide in particular in the production of a pharmaceutical composition for the treatment of a mammalian, including human disease, especially tumour diseases and ocular neovascularization.

viii) Another preferred embodiment of the invention relates also to an isolated nucleic acid molecule comprising a nucleotide sequence that encodes a polypeptide described in the preceding paragraphs i) – vi), especially such a polypeptide mentioned as being preferred, to a pharmaceutical composition comprising such a nucleic acid molecule and to the use of such a nucleic acid molecule in particular in the production of a pharmaceutical composition for the treatment of a mammalian, including human disease, especially tumour diseases and ocular neovascularization.

ix) In a further very preferred embodiment the invention relates to a recombinant vector, especially a recombinant viral vector such as a recombinant adenoviral vector, comprising a nucleic acid molecule described in the preceding paragraph viii), especially such a nucleic acid molecule mentioned as being preferred, to a pharmaceutical composition comprising such a recombinant vector and to the use of such a recombinant vector in particular in the production of a pharmaceutical composition for the treatment of a mammalian, including human disease, especially tumour diseases and ocular neovascularization, by means of gene therapy.

x) Another embodiment of the invention relates to a host cell comprising a recombinant vector as described in the preceding paragraph ix), especially such a recombinant vector mentioned as being preferred.

xi) The present invention also relates to a method for the treatment of a tumour disease in a mammal, including human, comprising: effecting an increase, in a mammal in need of such treatment, of the *in vivo* concentration of a polypeptide described in the preceding

paragraphs I) – VI), especially such a polypeptide mentioned as being preferred, to a tumour inhibiting effective amount, wherein said polypeptide has anti-tumour activity *in vivo*.

xii) The present invention further also relates to a method for the treatment of ocular neovascularization in a mammal, including human, comprising: effecting an increase, in a mammal in need of such treatment, of the *in vivo* concentration of a polypeptide described in the preceding paragraphs I) – VI), especially such a polypeptide mentioned as being preferred, to an ocular neovascularization inhibiting effective amount, wherein said polypeptide has anti-ocular neovascularization activity *in vivo*.

The invention most preferably relates to the sequences SEQ ID NO:1-8, especially the human sequences SEQ ID NO:3 [nucleotides (nt) 88-1702 of GeneBank Database Accession No. U07695; nucleotide sequence coding for the extracellular domain of human Eph B4], SEQ ID NO:4, SEQ ID NO:7 (nt 8-694 of GeneBank Database Accession No. U81262; nucleotide sequence coding for the extracellular domain of human ephrin B2) and SEQ ID NO:8, and to their use according to the present invention.

The polypeptides useful in the practice of the present invention (i.e., the polypeptides described in paragraphs I) – VI) above; hereinafter also referred to as "polypeptides of the invention") can be delivered to an individual in need of treatment using conventional pharmaceutical formulations.

An additional embodiment of the invention relates to the administration of a pharmaceutical composition, in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed above. Such pharmaceutical compositions contain as active ingredients the polypeptides of the invention or anti-idotypic antibodies to these polypeptides or mimetics of these polypeptides, said active ingredients being administered alone or in combination with at least one other agent, such as a stabilizing agent, in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones.

The pharmaceutical compositions encompassed by the invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-articular,

intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.).

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores may be used in conjunction with suitable coatings, such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, i.e., dosage.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations suitable for parenteral administration may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution,



Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers may also be used for delivery. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, emulsifying, encapsulating, entrapping, or lyophilizing processes.

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc.. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0.1-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. For administration of the polypeptides of the invention, such labeling would include amount, frequency, and method of administration.

Pharmaceutical compositions suitable for use in the invention include compositions where the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art.

A therapeutically effective dose of active agent can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model may also be

used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

A therapeutically effective dose refers to that amount of active ingredient, for example the polypeptides of the invention, which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED<sub>50</sub> (the dose therapeutically effective in 50% of the population) and LD<sub>50</sub> (the dose lethal to 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD<sub>50</sub>/ED<sub>50</sub>. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Normal dosage amounts, e.g., when an exogenously produced polypeptide of the invention is administered, may vary from about 0.1 to about 20 mg/kg per day, preferably from 2.5 to 20 mg/kg per day, depending upon the route and method of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.. Various biodegradable and biocompatible

polymeric matrices, including microcapsules, nanospheres, and implants, are useful in the practice of the present invention.

Microspheres are fine spherical particles containing active drugs. They are differentiated from nanospheres primarily by the size of the particle; microspheres have a diameter of less than approximately 1000  $\mu\text{m}$ , while nanospheres are submicronic ( $<1 \mu\text{m}$ ). Microsphere systems contain either homogeneous monolithic microspheres, in which the drug is dissolved or dispersed homogeneously throughout the polymer matrix, or reservoir-type microspheres, in which the drug is surrounded by the polymer matrix membrane shell. Monolithic and reservoir systems can also be combined. For instance, active drug can be dispersed within, or adsorbed onto, the polymer surface in a reservoir-type microsphere.

Biodegradable polymers can consist of either natural or synthetic materials that vary in purity. Natural polymers include polypeptides and proteins (e.g., albumin, fibrinogen, gelatin, collagen), polysaccharides (e.g., hyaluronic acid, starch, chitosan), and virus envelopes and living cells (e.g., erythrocytes, fibroblasts, myoblasts). Natural materials require cross-linking in the microencapsulation process, leading to the denaturation of the polymer and the embedded drug. As a result, synthetic polymers are most commonly used. Frequently used synthetic polymers include poly(-hydroxy) acids such as polylactic acid (PLA), polyhydroxybutyric acid, and copoly (lactic/glycolic) acid (PLGA). These compounds are biocompatible, lack immunogenicity, and have physical properties that permit them to be easily shaped (to control the bioerosion rate).

Colloidal particulate carriers can also be used in the methods of the present invention for delivering the polypeptides of the invention. Liposomes are the preferred colloidal vehicle, and are composed of a phospholipid bilayer that may act as a carrier for both hydrophilic and hydrophobic medications. Liposomes can be made from, e.g., neutral lipids, charged phospholipids, and cholesterol. The addition of an amphophilic polymer such as polyethylene glycol (PEG) onto the surface of a liposome can slow the clearance of liposomes.

Administration of the polypeptides of the invention modified with PEG is also within the scope of the present invention. PEGs are polymers comprised of repeating ethylene oxide subunits with two terminal hydroxyl groups that can be chemically activated. PEG molecules come in a number of different configurations. PEG chains include linear and branched structures in which one or more PEG chains are joined with linkers such as lysine or triazine. PEGs may be

attached, preferably covalently, to a polypeptide of the invention at a single site or at multiple sites. Since branched-chain PEGs attach at single or fewer sites than do linear PEGs, branched PEGs may be less likely to interfere with the biologic activity of the native molecule than would the attachment of multiple small linear-chain PEGs, and so are preferred. Pharmaceutical formulations suitable for oral administration of proteins are described, e.g., in U.S. Patents 5,008,114; 5,505,962; 5,641,515; 5,681,811; 5,700,486; 5,766,633; 5,792,451; 5,853,748; 5,972,387; 5,976,569; and 6,051,561.

The polypeptides of the invention can also be delivered to an individual in need of treatment using gene therapy methods. Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

In a preferred aspect, the therapeutic comprises a nucleic acid encoding a polypeptide of the invention that is part of an expression vector that expresses a polypeptide of the invention or a chimeric protein thereof in a suitable host. In particular, such a nucleic acid has a promoter operably linked to the polypeptide coding region, the promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, a nucleic acid molecule is used in which the polypeptide coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the desired nucleic acid.

Delivery of a nucleic acid encoding a polypeptide of the invention into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, a nucleic acid encoding a polypeptide of the invention is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, e.g., by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g., by infection using a defective or attenuated retroviral or other viral vector (see, e.g., U.S. Pat. No. 4,980,286 and others mentioned *infra*), or by direct injection of naked DNA (see, e.g., Blezinger et al., *Nature Biotechnology*, 17, 343-348 (1999)) or by use of microparticle

bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes (see, e.g., Chen et al., *Cancer Research*, 59, 3308-3312 (1999)), microparticles, or microcapsules, or by administering it in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see e.g., U.S. Patents 5,166,320; 5,728,399; 5,874,297; and 6,030,954) (which can be used to target cell types specifically expressing the receptors), etc.. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180; WO 92/22635; WO92/20316; WO93/14188; and WO 93/20221). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (see, e.g., U.S. Patents 5,413,923; 5,416,260; and 5,574,205).

In a specific embodiment, a viral vector that contains a nucleic acid encoding a polypeptide of the invention is used. For example, a retroviral vector can be used (see, e.g., U.S. Patents 5,219,740; 5,604,090; and 5,834,182). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. A nucleic acid encoding a polypeptide of the invention to be used in gene therapy is cloned into the vector, which facilitates delivery of the gene into a patient.

Adenoviruses are another type of viral vector that can be used in gene therapy. Adenovirus genomes are linear, double-stranded DNA molecules of approximately 36 kilobase pairs. Each extremity of the viral genome has a short sequence known as the inverted terminal repeat (or ITR), which is necessary for viral replication. The well-characterized molecular genetics of adenovirus render it an advantageous vector for gene transfer. Portions of the viral genome can be substituted with DNA of foreign origin. In addition, recombinant adenoviruses are structurally stable and no rearranged viruses are observed after extensive amplification.

Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver cells, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting

non-dividing cells. Methods for conducting adenovirus-based gene therapy are described in e.g., U.S. Patents 5,824,544; 5,868,040; 5,871,722; 5,880,102; 5,882,877; 5,885,808; 5,932,210; 5,981,225; 5,994,106; 5,994,132; 5,994,134; 6,001,557; and 6,033,8843.

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy using an adenoviral vector comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

The incorporation of genomic elements into the adenoviral vector may provide for enhanced expression of the DNA sequence encoding a polypeptide of the invention. Thus, in accordance with another aspect of the present invention, there is provided an adenoviral vector including at least one DNA sequence encoding a polypeptide of the invention, and at least one genomic element affecting the expression of such DNA sequence. The term "genomic element" is used as previously defined. Such genomic elements include, but are not limited to, introns, the 5' untranslated region, and the 3' untranslated region, and portions of the introns and 3' and 5' untranslated regions. The adenoviral vector may be as herein described. Promoters which control the DNA sequence may be selected from those described herein and from those known in the art.

The vector, consisting of infectious, but replication-defective, viral particles, which contain at least one DNA sequence encoding a polypeptide of the invention, is administered *in vivo* to a host in an amount effective to treat neovascularization or a tumour disease in the host. The host may be a mammalian host, including human and non-human primate hosts.

A viral vector can be administered in an amount of from about  $10^6$  plaque forming units (pfu) to about  $10^{14}$  plaque forming units, preferably from about  $10^6$  plaque forming units to about  $10^{11}$  plaque forming units, more preferably from about  $10^9$  plaque forming units to about  $10^{10}$  plaque forming units. Adenoviral vectors, in the quantities set out above, are preferred.

The infectious vector particles can be administered systemically, such as, for example, by intravenous administration (such as, for example, via peripheral vein injection) or administered via the portal vein, to the bile duct, intramuscularly, intraperitoneally, or intranasally. Alternatively,

Infectious vector particles can be administered locally, by, e.g., intraocular injection or injection at the site of a tumour. Intraocular injection can be either into the anterior or posterior chamber of the eye, e.g., into the aqueous humor or vitreous humor. Alternatively, the injection can be subretinal, e.g., by injection of aliquots (e.g., 1 to 10 microliters per aliquot) of vector-containing solution behind the retina, after which the solution is absorbed and the infectious vector particles infect local cells of the ocular tissues and produce the active polypeptide. Such administration can comprise either a single injection, multiple injections administered on the same day, single injections administered over a period of weeks or months, or multiple injections administered over a period of weeks or months.

The vector particles may be administered in combination with a pharmaceutically acceptable carrier suitable for administration to a patient. The carrier may be a liquid carrier (for example, a saline solution), or a solid carrier, such as, for example, microcarrier beads.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy, including e.g. endostatin gene therapy for tumours (see, e.g., Nguyen et al., Cancer Research, 58, 5673-5677 (1998)). Methods for producing and utilizing AAV are described, e.g., in U.S. Patents 5,173,414; 5,252,479; 5,552,311; 5,658,785; 5,763,416; 5,773,289; 5,843,742; 5,869,040; 5,942,496; and 5,948,675.

Other viral vectors which can be used in accordance with the present invention include especially gutless adenoviral vector, retroviral-based vectors such as Moloney murine leukemia virus and spleen necrosis virus, and other vectors derived from retroviruses such as Rous Sarcoma virus, Harvey sarcoma virus, avian leukosis virus, myeloproliferative sarcoma virus and mammary tumour virus. Lentiviral vectors are also preferred, especially bovine immunodeficiency viral vectors, such as those disclosed in PCT publication WO 01/44458.

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleotide sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc.. Numerous techniques are known in the art for the introduction of foreign genes into cells and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

Methods for the production and administration of such cells (i.e., those that produce high levels of protein from either endogenous or exogenous genes or nucleic acids) are described in, *inter alia*, U.S. Patent Nos. 5,641,670; 5,733,761; 5,968,502; 6,048,729; 6,054,288; 6,063,630; and 6,187,305. In a preferred embodiment, cells producing a polypeptide of the invention are delivered in microencapsulated form, e.g., in the form of cells microencapsulated in sodium alginate or calcium alginate poly L-lysine alginate (see, e.g., Read *et al.*, *Nature Biotechnology* 19, 29-34 (Jan 2001) and Joki *et al.*, *Nature Biotechnology* 19, 35-39 (Jan 2001)). The microencapsulated cells can be implanted proximally to the eye or the tumour, or at a site where a polypeptide of the invention produced by the cells will most quickly enter the subject's bloodstream, e.g., in the liver. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, e.g., as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc..

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In an embodiment in which recombinant cells are used in gene therapy, a nucleic acid encoding a polypeptide of the invention is introduced into the cells such that it is expressible by the cells or



their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem-and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention. Such stem cells include but are not limited to hematopoietic stem cells (HSCs), stem cells of epithelial tissues such as the skin and the lining of the gut, embryonic heart muscle cells, liver stem cells (see, e.g., WO 94/08598), and neural stem cells.

Epithelial stem cells (ESCs) or keratinocytes can be obtained from tissues such as the skin and the lining of the gut by known procedures. In stratified epithelial tissue such as the skin, renewal occurs by mitosis of stem cells within the germinal layer, the layer closest to the basal lamina. Stem cells within the lining of the gut provide for a rapid renewal rate of this tissue. ESCs or keratinocytes obtained from the skin or lining of the gut of a patient or donor can be grown in tissue culture. If the ESCs are provided by a donor, a method for suppression of host versus graft reactivity (e.g., irradiation, drug or antibody administration to promote moderate immunosuppression) can also be used.

With respect to HSCs, any technique which provides for the isolation, propagation, and maintenance *in vitro* of HSCs can be used in this embodiment of the invention. Techniques by which this may be accomplished include (a) the isolation and establishment of HSC cultures from bone marrow cells isolated from the future host, or a donor, or (b) the use of previously established long-term HSC cultures, which may be allogeneic or xenogeneic. Non-autologous HSCs are used preferably in conjunction with a method of suppressing transplantation immune reactions of the future host/patient. In a particular embodiment of the present invention, human bone marrow cells can be obtained from the posterior iliac crest by needle aspiration (see, e.g., Kodo et al., 1984, J. Clin. Invest. 73:1377-1384). HSCs can be made highly enriched or in substantially pure form. This enrichment can be accomplished before, during, or after long-term culturing, and can be done by any techniques known in the art. Long-term cultures of bone marrow cells can be established and maintained by using, for example, modified Dexter cell culture techniques (Dexter et al., 1977, J. Cell Physiol. 91:335) or Witlock-Witte culture techniques (Witlock and Witte, 1982, Proc. Natl. Acad. Sci. USA 79:3608-3612).

It is understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

The pharmacological useful properties of the polypeptides of the present invention as therapeutics against tumour diseases and ocular neovascularization can be demonstrated for example by using known animal models for said diseases, such as those described e.g. in the Examples below, and applying the polypeptides, nucleic acid molecules or recombinant vectors of the present invention in these models.

**Description of the Figures:**

**Fig. 1:** Growth of A375 tumours in the flanks of immunosuppressive mice, measured as tumour weight 14 days after application of tumour cells.

***Control:*** Tumours which develop within 14 days after application of  $1 \times 10^6$  A375 tumour cells, that are stably transfected with the empty pCDNA3 vector.

***s-EphB4:*** Tumours which develop within 14 days after application of  $1 \times 10^6$  A375 tumour cells, which are stably transfected with the pCDNA3 vector containing the coding sequence for the extracellular domains of the Eph B4 receptor and which secrete the soluble s-EphB4 polypeptide.

***s-ephrinB2:*** Tumours which develop within 14 days after application of  $1 \times 10^6$  A375 tumour cells, which are stably transfected with the pCDNA3 vector containing the coding sequence for the extracellular domains of the ephrin B2 ligand and which secrete the soluble s-ephrinB2 polypeptide.

**Fig. 2:** Growth of A375 tumours in the flanks of immunosuppressive mice.

Immunosuppressive mice are injected either in both flanks with A375 control tumour cells (animal group 1), or are injected in both flanks with A375 tumour cells which secrete s-EphB4 (animal group 2), or are injected in the left flank with A375 control tumour cells and in the right flank with A375 tumour cells which secrete s-EphB4 (animal group 3). The figure shows the tumour weight after 14 days (A) and the growth of the tumours (B). In the graph depicting the growth of tumours, for animal groups 1 and 2 the tumour volume of tumours from both flanks is determined.

**Examples:**

The following Examples serve to illustrate the invention without limiting its scope.

**Example 1: Inhibition of tumour angiogenesis and tumour growth by expression of the soluble extracellular domains of murine Eph B4 (murine s-EphB4) or ephrin B2 (murine s-ephrinB2) in tumour cells.**

**Cloning of murine s-EphB4 and s-ephrinB2 eukaryotic expression vectors:**

The nucleotide sequences coding for the soluble extracellular domains of murine Eph B4 (SEQ ID NO:1; nt 103-1719 of GeneBank Database Accession No. U06834) and murine ephrin B2 (SEQ ID NO:5; nt 161-847 of GeneBank Database Accession No. L38847) are amplified by means of sequence-specific oligonucleotide primers and Reverse Transcription Polymerase Chain Reaction (RT PCR) from isolated total RNA from mouse cerebral tissue or mouse kidney tissue, and modified with corresponding restriction cleavage sites as well as a stop codon. The amplified DNA is cloned by means of HindIII and BamHI into the vector pCDNA3 (Invitrogen) and the accuracy of the sequence is checked. The pCDNA3 vector contains the cytomegalovirus (CMV) promoter region and the G418 resistance gene. With this vector, the corresponding polypeptides s-EphB4 and s-ephrinB2 can be constitutively expressed in mammal cells under the control of the CMV promoter.

**Generation of stably transfected tumour cells, which express either s-EphB4 (SEQ ID NO:2) or s-ephrinB2 (SEQ ID NO:6):**

The recombinant vectors with the specific murine s-EphB4 and s-ephrinB2 nucleotide sequences, as well as the empty vector, are transfected with lipofectin (Gibco, LifeScience) into A375 human melanoma cells (ATCC No.: CRL-1619). Stable cell clones are generated by selection with 0.5 mg/ml G418 (Gibco, LifeScience). The expression of s-EphB4 and s-ephrinB2 is verified by Northern analysis and by a ligand receptor binding test [to this end, each 2 ml of conditioned tumour cell medium is concentrated by about five times (Amicon, Zentrifräp). 0.1 ml of concentrate is applied to a nitrocellulose membrane, and the membrane is subsequently incubated for one hour at room temperature in Tris-buffered saline (TBS) with 1% BSA. s-ephrinB2 is detected by incubation with biotinylated s-EphB4-Fc fusion protein (1 µg/ml; R&D Systems, Minneapolis, MN) and streptavidin coupled alkaline phosphatase. s-EphB4 is detected by incubation with biotinylated s-ephrinB2-Fc

fusion protein (1 µg/ml; R&D Systems, Minneapolis, MN) and streptavidin coupled alkaline phosphatase. Evidence of binding is found by enzymatic detection of the alkaline phosphatase]. In the proliferation assay, the transfected cell clones did not show differing growth kinetics (for the proliferation test, 8000 cells were seeded in a 24-well cell culture plate and the cell count was determined after 24, 48, 72 and 96 hours).

The influence of s-EphB4 and s-ephrinB2 on tumour growth and tumour angiogenesis:  $1 \times 10^6$  transfected A375 tumour cells are each injected intradermally into one or both flanks of immunosuppressive mice. Tumour growth is monitored over 14 days. After 14 days, the test animals are killed, the tumours prepared, weighed and examined histologically and by means of immunohistochemistry.

Monitoring of the body weight of the test animal, as well as observation of conspicuous behavioural patterns, are carried out to check for acute toxic side effects.

#### Results:

Animals which are injected with tumour cells (A375), which constitutively secrete either s-EphB4 or s-ephrinB2, develop considerably smaller tumours than animals which are injected with control cells, which are transfected with the empty vector (Fig. 1). The tumours, which are formed by A375 tumour cells and constitutively secrete either s-EphB4 or s-ephrinB2, have a disordered vessel structure compared to control tumours.

Animals that are injected with control tumour cells in both flanks develop tumours of equal size on both flanks. The kinetics of tumour growth is unchanged on both flanks (for the kinetics of tumour growth, the tumour volume (greatest diameter and perpendicular on this diameter) is measured by a slide gauge. The volume is determined by the factor  $0.5 \times \text{tumour diameter [mm]} \times \text{perpendicular}^2 [\text{mm}^2]$ ). Animals which are injected in both flanks with s-EphB4 secreting tumour cells, develop tumours of equal size on both sides, but the tumour size is significantly smaller than that of the corresponding control tumours. The kinetics of tumour growth is unchanged on both flanks.

Animals which are injected with control tumour cells in the left flank and s-EphB4 secreting tumour cells in the right flank, develop tumours of different size. On the left flank, considerably smaller control tumours develop, compared with the control tumours of animals which are injected with control cells in both flanks (Fig. 2 A, B).

In the groups of test animals, conspicuous behaviour patterns, such as motor disorders or hypermotor activities, are not observed, nor noticeable weight loss.

**Example 2: Therapeutical application of recombinant s-EphB4 (SEQ ID NO:2) and s-ephrinB2 (SEQ ID NO:6).**

The cDNA sequences coding for the soluble extracellular domains of the murine Eph B4 receptor (SEQ ID NO:1) or for those of the murine ephrin B2 ligand (SEQ ID NO:5), are cloned into a eukaryotic expression vector (for example pCDNA3, see also Example 1). "Chinese Hamster Ovarian" (CHO) cells are transfected with these recombinant vectors and stable cell clones are isolated by selection markers.

The expression levels of secreted soluble s-EphB4 protein and s-ephrinB2 protein from stably transfected CHO cell clones are determined by Western Blot Analysis (anti-EphB4 antibodies, Santa Cruz Ltd; anti-EphrinB2 antibodies, R&D Systems, Minneapolis, MN). Functionality is tested in a binding test, in which cell supernatants of stably transfected CHO cells are applied to a nitrocellulose membrane, the membrane is subsequently incubated for one hour at room temperature in TBS with 1% BSA. s-ephrinB2 is detected by incubation with biotinylated s-EphB4-Fc fusion protein (1 µg/ml, R&D Systems, Minneapolis, MN) and streptavidin coupled alkaline phosphatase. s-EphB4 is detected by incubation with biotinylated s-ephrinB2-Fc fusion protein (1 µg/ml, R&D Systems, Minneapolis, MN) and streptavidin coupled alkaline phosphatase. Evidence of binding is found by enzymatic detection of the alkaline phosphatase. CHO cell clones which noticeably secrete functionally active s-EphB4 protein or s-ephrinB2 protein are fermented and the recombinant proteins from the cell supernatant are purified. Purification is carried out by column chromatography with ConcavalinA affinity chromatography (Amersham/Pharmacia) followed by DEAE sepharose (Amersham/Pharmacia). Separation according to size is then carried out by gel filtration. The purified protein is dissolved in "Hanks buffered salt solution" (HBSS), sterile-filtered and stored in lyophilised state. Directly before therapeutical usage, the protein is dissolved in e.g. sterile water in a concentration of 2 mg/ml.

**Therapy test:**

A375 tumours are induced by intradermal injection of  $1 \times 10^6$  A375 melanoma cells into the flanks of immunosuppressive mice. Animals which develop a readily tangible tumour are treated by daily intraperitoneal injection of 0.1 mg/animal of recombinant protein and compared with tumour-bearing control animals which are given the same intraperitoneal

volume of HBSS. The success of therapy is determined by the tumour growth and histochemical and immunohistochemical characterization.

Monitoring of the body weight of the test animal, as well as observation of conspicuous behavioural patterns, are carried out to check for acute toxic side effects.

**Results:**

A375 tumour-bearing, immunosuppressive mice, which receive injections of recombinant, purified s-EphB4 polypeptide or s-ephrinB2 polypeptide, develop considerably smaller tumours with disordered vessel structure compared to control animals which are given corresponding volumes of HBSS.

In the groups of test animals, conspicuous behaviour patterns, such as motor disorders or hypermotor activities, are not observed, nor noticeable weight loss.

**Example 3: Generation and characterization of an adenoviral vector containing the DNA encoding for the soluble extracellular fragment of the human Eph B4 receptor.**

Construction of adenoviral (Av3) vector expressing the extracellular domain of human Eph B4 receptor (human s-EphB4):

The cDNA encoding the extracellular domain of human Eph B4 is PCR-amplified from human fetus whole Marathon-Ready cDNA (Clontech Laboratories, Inc. Palo Alto, CA) with the primers; sense (5'- ATC ACT GCT AGC AAC TCA GTT CGG ATC CTA CC-3') and antisense (5'- ATC ATA GCT AGC TCA CTG CTC CCG CCA GCC CTC GC -3'). The PCR amplifies the region encoding the first 537 amino acids coding region of human Eph B4 (aa 1-537 of SEQ ID NO:4), including the signal peptide (the first 15 amino acids) and extracellular region of the mature Eph B4 receptor protein (16 to 537 amino acids). PCR is carried out with Pfu DNA polymerase (Stratagene, La Jolla, CA) in the presence of 1  $\mu$ M primers as described, 0.4 mM dNTP, 1.5 mM MgCl<sub>2</sub> and 1 ng cDNA. The reaction is run for 35 cycles with the following conditions: 94 °C hot start for 1 min, 95 °C denaturation for 1 min, 62.5 to 67.5 °C with 0.5 °C thermal gradient annealing for 1 min, 72 °C extension for 3 min, and ended at 72 °C extension for 10 min. The 1674 base pairs (bp) s-EphB4 containing DNA fragments are pooled together, gel-purified and subcloned into pPCR-script plasmid according to the manufacturer's procedure (Stratagene, La Jolla, CA). The NheI digested s-EphB4 DNA fragment is gel purified and cloned into the SpeI site of the adenoviral shuttle plasmid, pAvsCvXix [this vector contains the left end of the adenoviral vector

including the left inverted terminal repeat (ITR), a CMV promoter, an adenovirus major late promoter tripartite leader sequence, a multiple cloning site for inserting the transgene of interest, a SV40 early polyadenylation signal, and a loxP site], downstream of the simian CMV promoter and upstream of the SV40 polyadenylation signal to generate pAvCshs-EphB4-C16. The DNA sequence of the entire PCR-amplified region of the pAvCshs-EphB4-C16 is confirmed by sequence analysis by the Gene Therapy Core Technologies Molecular Core Laboratory.

The recombinant adenoviral vector Av3Cshs-EphB4 (with E1, E2a, and E3-deletions) is generated in the following manner. The pAvCshs-EphB4-C16 and pSQ3 (a 31'574 bp plasmid vector containing the right hand portion of the adenoviral vector genome and a loxP site that allows Cre-mediated recombination with pAvCshs-EphB4-C16) are first linearized with NotI and ClaI restriction enzymes, respectively. A transient transfection is performed on a 6-well plate containing  $5 \times 10^6$  S8 cells [S8 cells are derived from A549 cells (ATCC No. CCL-185) that have been transfected with plasmids containing the E1 and E2a regions, respectively, of the adenoviral vector (see also: Gorziglia et al., J. Virology 70, 4173-4178, 1996] per well using the LipofectAMINE PLUS reagent (Life Technologies, Rockville, MD) according to the manufacturer's procedure. The cells are cultured with media in the presence of  $0.3 \mu\text{M}$  of dexamethasone the day before transfection. The DNA-PLUS is prepared with  $0.5 \mu\text{g}$  of linearized pAvCshs-EphB4-C16,  $1 \mu\text{g}$  of linearized pSQ3 and  $0.5 \mu\text{g}$  of the expression plasmid pC-Cre3.1 encoding the Cre recombinase, in a  $100\text{-}\mu\text{l}$  Optimen (Life Technologies) with  $6\text{-}\mu\text{l}$  of PLUS reagent. After 15 min of incubation, the DNA-PLUS is mixed with  $0.8\text{-ml}$  of 1 to 200-fold diluted lipofectAMINE Reagent. The DNA-PLUS-LipofectAMINE complex is added to the cells and incubated for 3 hours. The medium is then replenished with fresh medium. After seven to ten days incubation, cytopathic effect (CPE) is observed. The cells and the medium are harvested by scraping. A crude viral lysate (CVL) is prepared from 1 ml resuspended cell pellet by five cycles of freezing and thawing. The Av3Cshs-EphB4 vector is re-amplified in S8 cells with  $0.3 \mu\text{M}$  dexamethasone in Richter's CM medium containing 5% FBS until CPE was observed. The CVL is plaque-purified. The harvested single plaque free of replication competent adenovirus (RCA) is selected to generate purified vector.

**Av3Cshs-EphB4 research seed lot characterization:**

The research seedlot purified Av3Cshs-EphB4 vector is scaled up by the GTI Gene Therapy Core Technologies. The adenoviral vector titer (particles/ml) and biological titer (pfu/ml) are determined as described (Mittereder, N., March, K.L., and Trapnell, B.C. (1996) *J. Viro.* 70, 7498-7509) by GTI Gene Therapy Core Technologies. The ratio of total particles to infectious particles (particles/pfu) is calculated.

**Characterization of recombinant Av3Cshs-EphB4:**

Genomic DNA is prepared from the purified Av3Cshs-EphB4 vector using DNA Isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's procedure. Various restriction digests by BamHI, Xba1, and XhoI of the purified viral DNA of Av3Cshs-EphB4, are compared on 1.0% agarose-TAE gel and stained with ethidium bromide to visualize the various digests for the verification of the correct genome structure and purity.

**Northern blot analysis:**

Hep3B (ATCC No. HB-8064) and A549 cells are transduced with Av3Cshs-EphB4, or the control Av3CNull (same genome structure with no transgene in the expression cassette) vectors at the particles to cell ratio of 750. 72 hours after transduction, the cell pellets are harvested. Total RNA is isolated using the RNA-DNA STAT 60 (Tel-Test) extraction method. Northern blot analysis is carried out according the standard procedure. A 20 µg aliquot of total RNA is analyzed on 1% agarose formaldehyde/MOPS gel. RNA is transferred to a nylon membrane and prehybridized in the solution of 6.75% SDS, 0.5M NaPO<sub>4</sub>, pH 7.0, 1.0 mM EDTA, pH 8.0, 5 µg/ml BSA, and 100 µg/ml sheared, denatured salmon sperm DNA. The probe of 1368 bp s-EphB4 internal fragment is generated by EcoN1 digestion, gel purified and <sup>32</sup>P-labeled. The membrane is hybridized with a [<sup>32</sup>P]-labeled s-EphB4 internal probe at 68 °C overnight and washed in SSC/SDS containing buffers at 68 °C following the standard protocol. The membrane is exposed to an x-ray film at -70 °C overnight. The membrane is reprobed with [<sup>32</sup>P]-labeled Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal probe to verify equal amount of RNA loaded on each lane.

**SDS-PAGE and Western blot analysis:**

S8 and Hep3B cells are transduced with Av3Cshs-EphB4 or the control, Av3CNull vector at the particles to cells ratio of 100 and 500, respectively. 72 hours post transduction, the



conditioned media are collected and subjected to SDS-PAGE analyses. The resolved protein bands are then electrotransferred to PVDF membrane using the Mini Trans-blot Electrophoretic Transfer Cell (Cat# 165-3165, Bio-Rad). Western blot analyses are carried out according to the following procedure. Briefly, the membrane blot is blocked with blocking buffer (5% powdered milk, 0.1% Tween-20 and PBS buffer) for 30 min, probed with 1:1000 diluted rabbit anti-EphB4 antiserum (generated at GTI through outside service by Affinity Bioreagents Inc., Golden, CO) in blocking buffer at 4 °C overnight, probed with 1:5000 diluted Horse Radish Peroxidase (HRP)-conjugated anti-rabbit IgG antibody in TPBS (0.1% Tween-20 and PBS buffer) (Amersham Pharmacia Biotech) at 4 °C for 1 hour and developed in chemiluminescence detection mixture (ECL kit, Amersham Pharmacia Biotech) for 1 min followed by x-ray film exposure.

Polyclonal anti-EphB4 antiserum is generated according to the following procedure. The rabbits are immunized with KLH conjugated synthetic peptide of (C)DGQWEELSGLDDEEQH (amino acids 39-43 of internal region of human Eph B4) followed by two boost injections every other month. Sera are collected and confirmed with reactivity directed against peptide antigen by ELISA and against Eph B4 protein by Western blot using purified recombinant Mouse EphB4/Fc chimera protein (Cat#446-B4, R&D Systems, Minneapolis, MN).

**Solid phase EphB4-ephrinB2 binding assay:**

The solid phase assay is carried out as described in the following procedure. Briefly, mouse EphB4/Fc chimera (Cat#446-B4-200, R&D Systems) is immobilized on a 96-well Nunc-Immuno™ plate with polySorp™ surface (Fisher) at 4 °C overnight. After 4 washes with TPBS (0.5% Tween-20 in DPBS), the plate is blocked with 10% FBS in DPBS for 30 min followed by 6 washes with TPBS. Various concentration of biotinylated mouse ephrinB2/Fc chimera (Cat#BT496, R&D Systems) in DPBS is then incubated with immobilized mouse EphB4/Fc chimera for 1 to 2 hours. After extensive washes with TPBS, the plate is incubated with 100 µl of 0.23 µg/ml of Avidin-HRP (Cat#21123, Pierce) for 1 hour followed by development in the Horseradish Peroxidase substrate mixture of 3, 3', 5, 5'-tetramethylbenzidine solution and hydrogen peroxide (Cat # 172-1064, Bio-Rad, Hercules, CA) for 10 to 30 min. The colored product is determined by absorbance at 415 nm by an ELISA reader. To determine the inhibitory effect of s-EphB4, 100 ng/ml (2.1 nM) of biotinylated mouse ephrinB2/Fc chimera is incubated for 30 min to 1 hour with increasing amounts of conditioned medium from untreated control S8 cells or S8 cells transduced with either null vector or s-EphB4 expressing vector prior to incubation of the mixture with immobilized

EphB4/Fc. Data is analyzed using Prism nonlinear regression curve fit to determine the best-fit curve and Kd, concentration of ligand required to reach half-maximal binding.

#### **Matrigel Implant Assay:**

Liquid Matrigel (a sterile extract of basement membrane proteins) is obtained from Fisher Scientific, Part number CB40234C, mixed with test substances and preloaded into a 1.0 ml tuberculin syringes with G27 needles. Male nude mice (Harlan) weighing about 20 g are anesthetized and 0.45 ml of the undiluted Matrigel alone or the undiluted Matrigel containing 1 µg/ml of FGF-2 (Fibroblast Growth Factor 2) or 3 µg/ml of VEGF (Vascular Endothelial Growth Factor, R&D Systems, Minneapolis, Minnesota) and  $5 \times 10^5$  S8 cells transduced at 100 MOI (multiplicity of infection) with either null vector or s-EphB4 expressing vector are injected subcutaneously into the dorsal caudal portion of the midline (1 injection/animal) using a G27 needle. For VEGF-induced angiogenesis, 20 U/ml heparin is also included. The Matrigel will rapidly form a solid gel that persists for over 7 days. On day 7, all animals are euthanized by CO<sub>2</sub> and Matrigel plugs are harvested for histological analysis and hemoglobin measurement.

#### **Hemoglobin Analysis:**

Matrigel plugs are snap frozen with dry ice and dried over night. The dry weight is recorded and the plug rehydrated with 0.5 ml of 0.5% Tween-20 and water. After homogenization, the mixture is centrifuged at 14,000 rpm in a desktop centrifuge (Eppendorf 5415C, Brinkmann Instruments, Inc, Westbury, NY) for 30 min. Supernatant fluid is collected and its absorbance read at 405nm and converted to µg hemoglobin per mg Matrigel using a standard curve generated with hemoglobin standards. Data represent mean + SEM, n = 10. Experiments are done at least twice.

#### **Results:**

The gene therapy vector, Av3Cshs-EphB4 mediates s-EphB4 expression and secretion: Northern blotting analysis indicates that Av3Cshs-EphB4 transduced Hep3B and A549 cells express a 1.8 Kb mRNA transcript that specifically hybridizes to the Eph B4 cDNA probe. In Av3Cshs-EphB4 transduced S8 (without dexamethasone) and Hep3B cells, Western blot analysis also reveals a band with an apparent molecular mass of 85 kDa. This band is not observed in cells transduced with Av3CsNull or in untreated cells. The predicted molecular

mass of the expressed s-EphB4 protein is considerably smaller (57 kDa), suggesting that at least one of the three potential glycosylation sites is glycosylated.

**Inhibition of EphB4-ephrinB2 binding by s-EphB4:**

In the solid phase assay, dose-dependent saturation binding is demonstrated with the best curve-fit equation of  $\text{Absorbance} = \text{AbsMax} \times [\text{biotinylated ephrinB2}] / (\text{Kd} + [\text{biotinylated ephrinB2}])$  revealing a dissociation constant of  $1.16 \pm 0.24$  nM. AbsMax represents the absorbance at saturated biotinylated ephrinB2 concentration. The binding assay is then used to determine if s-EphB4 can effectively interfere with this binding. As shown in Table 1, conditioned media from untreated S8 cells and S8 transduced with null vector actually enhance the binding of Eph B4 with ephrin B2. In contrast, conditioned media prepared from Av3Cshs-EphB4 transduced S8 substantially inhibit the binding in a dose-dependent manner. These results indicate that this gene therapy vector can direct the expression of the extracellular domain of the Eph B4 receptor and can block the interaction of Eph B4 with its cognate partners.

**Adenoviral vector expressing the extracellular domain of Eph B4 can inhibit both VEGF and FGF-2 directed angiogenesis:**

As shown in Table 2, VEGF and FGF-2 are both able to substantially induce angiogenesis as measured by the increase in hemoglobin content in the Matrigel plug. However, FGF-2 is a more potent inducer of angiogenesis in this model (Table 2). Histological analysis reveals that the increase in hemoglobin content correlates with an increase in the number of blood vessels. The addition of Av3Cshs-EphB4 transduced cells substantially inhibits both VEGF- and FGF-2-induced angiogenesis. These results indicate that the soluble, extracellular domain of Eph B4 can inhibit angiogenesis induced by VEGF or FGF-2.

**Table 1: Inhibition of EphB4-ephrinB2 binding by gene therapy vector-mediated expression of the soluble, extracellular domain of Eph B4**

%Conditioned media	Absorbance at 415 nm		
	Untreated S8 cells	S8/Av3CsNull	S8/Av3Cs-hs-EphB4
0	0.63 ± 0.06	0.52 ± 0.11	0.60 ± 0.06
12.5	0.74 ± 0.06	0.55 ± 0.09	0.43 ± 0.07
25	0.89 ± 0.11	0.81 ± 0.14	0.41 ± 0.03
50	0.81 ± 0.15	0.76 ± 0.10	0.31 ± 0.02
100	0.97 ± 0.03	0.90 ± 0.26	0.29 ± 0.01

**Table 2: Inhibition of *in vivo* angiogenesis by gene therapy vector-mediated expression of the soluble, extracellular domain of Eph B4**

Treatment	Hemoglobin content/ Matrigel [µg/mg]	SEM	% Inhibition
Matrigel alone	0.48	0.06	
VEGF + null vector	4.24	0.46	
VEGF + s-EphB4	2.47	0.31	47%
FGF + null vector	12.85	1.68	
FGF + s-EphB4	9.01	1.26	31%

**Example 4: Application of Av3Csms-EphB4 or Av3Csms-ephrinB2 vector in a mouse tumour model.**

The recombinant adenoviral vectors Av3Csms-EphB4 and Av3CshsephrinB2 which contain the DNA encoding the soluble extracellular fragment of murine EphB4 and ephrinB2, respectively, are prepared analogously to the preparation of Av3Cshs-EphB4 as described in Example 3 above using the murine s-EphB4 and s-ephrinB4 cDNAs, respectively, instead of the human s-EphB4 cDNA.

**Gene therapy:**

A375 tumours are induced by intradermal injection of  $1 \times 10^6$  A375 melanoma cells into the flanks of immunosuppressive mice. Animals which develop a readily tangible tumour are treated by intravenous injection of Av3Csms-EphB4 or Av3CshsephrinB2 ( $1 \times 10^8$  "plaque forming units" (pfu)/animal –  $1 \times 10^9$  pfu/animal) and compared with tumour-bearing control animals which are treated with recombinant control adenoviruses (same genome structure with no transgene in the expression cassette) vectors. The success of therapy is determined by the tumour growth. The resulting tumours are further examined by histochemistry and immunohistochemistry.

Monitoring of the body weight of the test animal, as well as observation of conspicuous behavioural patterns, are carried out to check for acute toxic side effects.

**Results:**

A375 tumour-bearing, immunosuppressive mice which receive recombinant adenoviruses, which contain either the coding sequence for the extracellular domains of the murine Eph B4 receptor or that of the murine ephrin B2 ligand, develop substantially smaller tumours with a disordered vessel structure compared to control animals which receive control adenoviruses.

In the groups of test animals, conspicuous behaviour patterns, such as motor disorders or hypermotor activities, are not observed, nor noticeable weight loss.

**What is claimed is:**

1. An isolated soluble polypeptide which comprises an amino acid sequence of an extracellular region of an Eph B receptor or an essentially similar amino acid sequence thereof, said isolated soluble polypeptide being capable of binding with high affinity to a mammalian ephrin B ligand.
2. A polypeptide according to claim 1 which comprises an amino acid sequence of an extracellular region of Eph B4 or an essentially similar amino acid sequence thereof, said polypeptide being capable of binding with high affinity to a mammalian ephrin B ligand.
3. A polypeptide according to claim 1 which comprises SEQ ID NO:2 or SEQ ID NO:4, or an essentially similar amino acid sequence thereof, said polypeptide being capable of binding with high affinity to a mammalian ephrin B ligand.
4. An isolated soluble polypeptide which comprises an amino acid sequence of an extracellular region of an ephrin B ligand or an essentially similar amino acid sequence thereof, said isolated soluble polypeptide being capable of binding with high affinity to a mammalian Eph B receptor.
5. A polypeptide according to claim 4 which comprises an amino acid sequence of an extracellular region of ephrin B2 or an essentially similar amino acid sequence thereof, said polypeptide being capable of binding with high affinity to a mammalian Eph B receptor.
6. A polypeptide according to claim 4 which comprises SEQ ID NO:6 or SEQ ID NO:8, or an essentially similar amino acid sequence thereof, said polypeptide being capable of binding with high affinity to a mammalian Eph B receptor.
7. A pharmaceutical composition comprising a polypeptide according to any one of claims 1 to 6.
8. Use of a polypeptide according to any one of claims 1 to 6 in the production of a pharmaceutical composition for the treatment of a tumour disease of mammals, including humans.

9. Use of a polypeptide according to any one of claims 1 to 6 in the production of a pharmaceutical composition for the treatment of ocular neovascularization in mammals, including humans.
10. An isolated nucleic acid molecule comprising a nucleotide sequence that encodes a polypeptide according to any one of claims 1 to 6.
11. A pharmaceutical composition comprising a nucleic acid molecule according to claim 10.
12. Use of a nucleic acid molecule according to claim 10 in the production of a pharmaceutical composition for the treatment of a tumour disease of mammals, including humans.
13. Use of a nucleic acid molecule according to claim 10 in the production of a pharmaceutical composition for the treatment of ocular neovascularization in mammals, including humans.
14. A recombinant vector comprising a nucleic acid molecule according to claim 10.
15. A recombinant vector according to claim 14, which is a recombinant viral vector.
16. A recombinant viral vector according to claim 15, which is a recombinant adenoviral vector.
17. A recombinant viral vector according to claim 15, which is a recombinant lentiviral vector.
18. A recombinant viral vector according to claim 15, which is a recombinant bovine immunodeficiency virus (BIV) vector.
19. A pharmaceutical composition comprising a recombinant vector according to any one of claims 14 to 18.

20. Use of a recombinant vector according to any one of claims 14 to 18 in the production of a pharmaceutical composition for the treatment of a disease of mammals, including humans, by means of gene therapy.

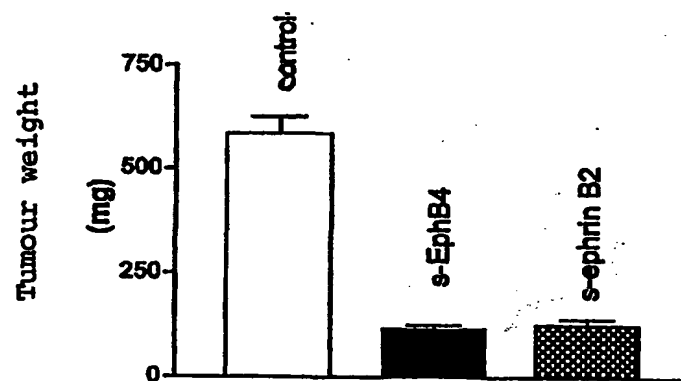
21. Use according to claim 20 for the treatment of a tumour disease of mammals, including humans.

22. Use according to claim 20 for the treatment of ocular neovascularization in mammals, including humans.

23. A host cell comprising a recombinant vector according to any one of claims 14 to 18.

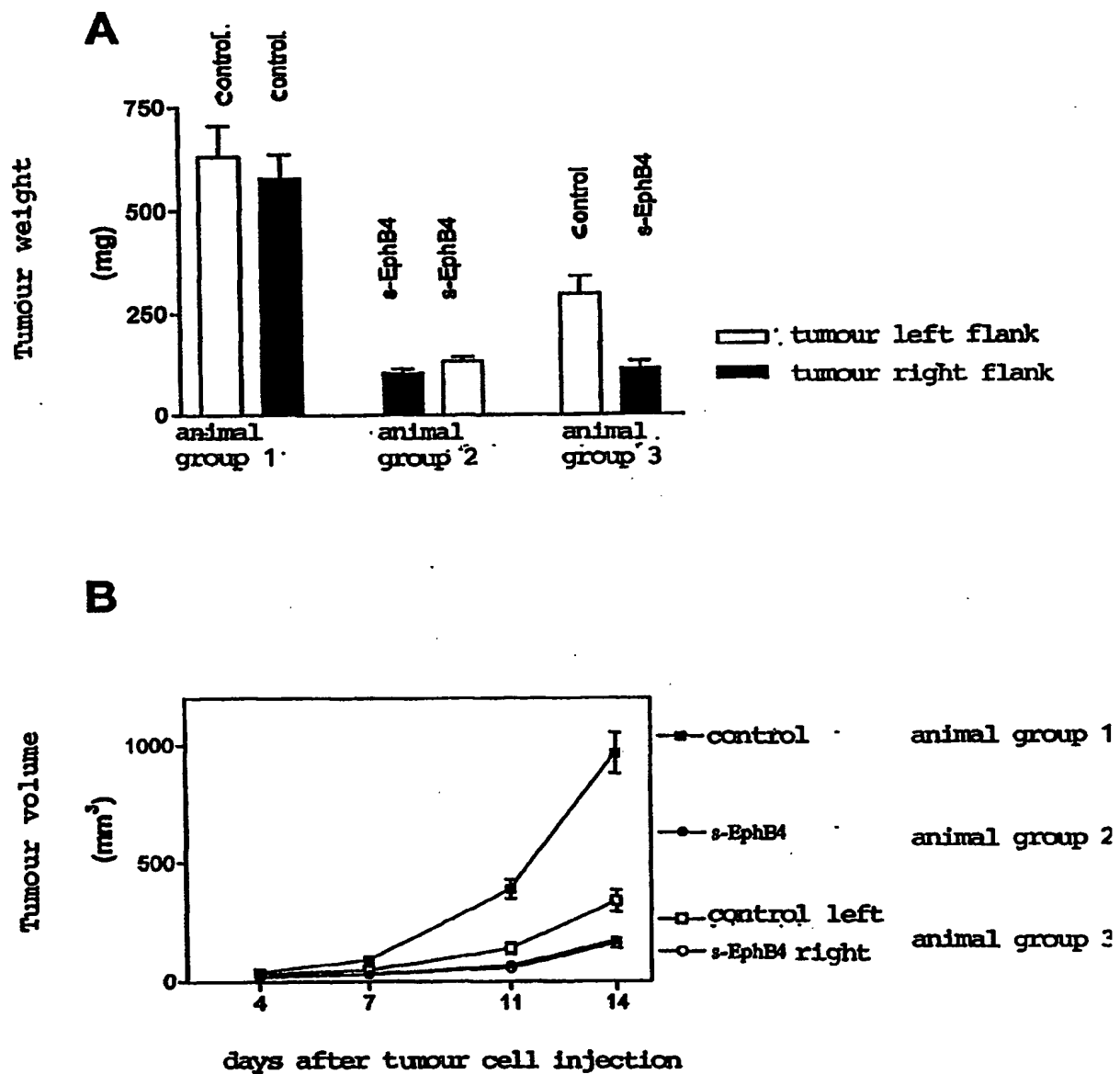


Fig. 1



2/2

Fig. 2



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Cys Pro Lys Val Asp Ser Lys Thr Val Gly Gln Tyr Glu Tyr Tyr Lys  
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Pro Leu Leu Asn Cys Ala Lys Pro Asp Gln Asp Ile Lys Phe Thr Ile  
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Lys Phe Gln Glu Phe Ser Pro Asn Leu Trp Gly Leu Glu Phe Gln Lys  
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Leu Asp Asn Gln Glu Gly Gly Val Cys Gln Thr Arg Ala Met Lys Ile  
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210 215 220

Val Ala Leu Phe Ala  
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# INTERNATIONAL SEARCH REPORT

Inventor's Application No  
PCT/EP 01/11252

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/715 C07K14/52 C12N5/10 A61K48/00 A61K38/19

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data, SEQUENCE SEARCH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 670 625 A (BECKMANN M PATRICIA ET AL) 23 September 1997 (1997-09-23)  see whole doc. esp. examples, claims and col. 31.9ff, col 9 1.34 ff.	4, 7, 10, 11, 14, 19, 20, 23
X	WO 96 26958 A (HARVARD COLLEGE) 6 September 1996 (1996-09-06)  see whole doc. esp. claims and p. 5, 1.14, p. 8, 1.13 ff.	4, 5, 7, 8, 10-12, 14, 19, 20, 23
X	WO 99 17796 A (BRISKIN MICHAEL J ; LEUKOSITE INC (US); ZOU LILY (US)) 15 April 1999 (1999-04-15) see whole doc. esp. claims, and e.g. p. 21.18; p. 4, 1.8-21,	4, 8, 10, 11, 14, 19, 20, 23
	-/-	

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☒ Patent family members are listed in annex.

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- \*P\* document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search

10 January 2002

Date of mailing of the international search report

18/01/2002

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Mueller, F

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 01/11252

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 00 30673 A (GENENTECH INC) 2 June 2000 (2000-06-02) see whole doc. esp. claims and p.10, l.24-39; p.16, l.21-28; p.22, l.36-p.28., l.12)</p>	1-23
X	<p>WO 96 02645 A (GENENTECH INC) 1 February 1996 (1996-02-01)  see whole doc. esp. claims and seq ids 2,4 and 6</p>	4,7,10, 11,14, 19,20,23
X	<p>WO 96 01839 A (IMMUNEX CORP) 25 January 1996 (1996-01-25)  see whole doc. esp. <u>claims</u> and seq id 2</p>	4,6,7, 11,14, 19,20,23

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In International Application No  
PCT/EP 01/11252

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